THE EGG COCOONS OF SCOLOPLOS ARMIGER O. F. MÜLLER

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In his review entitled "La ponte et l'incubation chez les annélides polychètes," Gravier (1923) listed 12 species which are known to lay their eggs in an agglomeration but which do not incubate them. Several more species have since been shown to spawn similarly (Herpin, 1925; Aiyar, 1931; Wilson, 1932; Day, 1934; Thorson, 1946; Bookhout and Horn, 1949; Rullier, 1954; Pillai, 1958) but no study of the chemical and physical nature or ecological role of the egg cocoons seems to have been published. The present paper is concerned chiefly with the spawning of *Scoloplos armiger* and the chemical nature of the cocoon jelly.

S. armiger has the same general distribution over the shore as *Arenicola marina* (Thamdrup, 1935; Linke, 1939). The egg cocoons are found where the worms normally live, on the gently sloping muddy sand of the foreshore which does not dry out at low tide. They have often been described, for example by Cunningham and Ramage (1888), Ehlers (1892), de Groot (1907), Thamdrup (1935), Linke (1939), Thorson (1946) and Smidt (1951). At the time of formation the cocoon is a pear-shaped or globular mass of firm jelly, about 1 to 1.5 cm. in diameter and containing from 500 to 1200 eggs, and is inserted into the sand by a stalk about 6 cm. long. Since a mature female may contain between 3000 and 5000 eggs, it looks as if a worm may form more than one cocoon (Smidt, 1951).

SPAWNING

Numerous authors, for example Mau (1882), Thorson (1946) and Anderson (1959), state that spawning occurs in the spring and lasts for about a month, its exact dates varying with the particular district. At Whitstable spawning takes place in February or March and lasts little more than a week. The method of forecasting the spawning period by the measurement of oöcytes showed, during the winter 1949–50, that spawning might be expected in February, 1950. Its onset occurred on February 18, 1950, with a suddenness which is shown by the following observations.

During a careful scrutiny of a wide area of the shore at Whitstable, lasting from 08.00 to 10.00 hr. on 18 February, only 5 (total) cocoons were seen. After the shore had been covered by the midday high tide an average of two to three cocoons *per square meter* was counted in the same locality. At 10.00 hr. on February 19 cocoon counts gave an average of 5 per sq. m. but by the evening low tide of the same day the count had risen to an average of 23 per sq. m.

In other years spawning began on the days listed and coincided with the spring tides of new (●) or full (○) moon. 1949, 15 Feb. ○; 1950, 18 Feb. ●; 1952, 29 Feb. ●; 1953, 1 Mar. ○; 1954, 20 Feb. ○; 1955, 10 Mar. ○; 1956, 14 Mar. ●.

In 1964 spawning occurred at Whitstable on 29 Feb. [illegible] (Gibbs, private communication) while at Southend-on-Sea, on the opposite side of the Thames Estuary, a few worms spawned in February but the massive spawning did not occur until about 24–25 March.

Formation of the cocoon

Cocoon formation has not been achieved in the laboratory and is difficult to observe on the shore, as it occurs at high tide. It is not known how many worms take part in the formation of a cocoon but indirect evidence suggests that male and female worms come together in a burrow during spawning so that the eggs are fertilized as they leave the terminal glandular portions of the nephridia of the female. It is these organs which secrete the jelly which envelops the worms and their eggs while they are still within the burrow. On one occasion two worms were found within the stalk of a new cocoon and a search in the sand immediately below newly-formed cocoons usually revealed male and female worms occurring close together. The sex ratio, male to female, is approximately 1.3:1.

The shape of the cocoon, that of a single large drop of a viscous substance emerging from a tube, is clearly due to its method of formation. Rarely are malformed cocoons found although occasionally two smaller drops are attached to a single stalk and more rarely an additional stalk is found, as if one of the worms entered or left the cocoon by a different route. On one occasion male and female worms spawned in the laboratory soon after collection and before they had established their proper burrows. The cocoon jelly was released below the surface of the sand in irregular masses, emphasizing that the form of the cocoon normally results from its method of formation as a drop emerging from a tube.

De Groot (1909) stated that copulation occurs, but Anderson (1959) was unable to find sperm or fertilized eggs in the coelom of the female. Observations made on stained sections of fixed ripe females confirm this; neither were living spermatozoa seen in the jelly of freshly-laid cocoons although the eggs which they contained were fertilized. It looks, therefore, as if the eggs are fertilized immediately they leave the body cavity of the female before the jelly has had time to swell.

THE COCOON JELLY

Source of the material

The jelly of which the cocoon is formed is provided by the female and comes from the specialized end portions of the nephridia, of which about 100 pairs in the middle region of the body develop conspicuous white swellings at the bases of the notopodia (Eisig, 1914). These glandular portions surround the nephridiopores and consist of very large cells, each of which is greatly distended in the breeding season with aggregations (measuring 20–30 μ in diameter) of rod-like bodies, each 5–10 μ long (Fig. 1).

Formation of jelly from rodlets

When temporary preparations of the glandular nephridial cells are made and squashed or teased in sea water, some aggregations of rodlets may be forced out intact but many are broken up into their constituent rodlets. In such a preparation some of the rodlets appear to explode suddenly and at random but a rodlet can sometimes be seen to swell a little before suddenly bursting. Under phase-contrast illumination the rodlets are sufficiently dense to appear quite black but the diffuse body formed after bursting can only just be made out. No ghosts or envelopes of rodlets are left, so that they appear to be simply a concentrated form of the jelly material which is able to absorb water and greatly to increase in volume.

The effect on the rodlets of various reagents was watched, using phase-contrast illumination, during irrigation under a cover glass or in hanging drop preparations. The tests were made about two weeks before spawning took place.

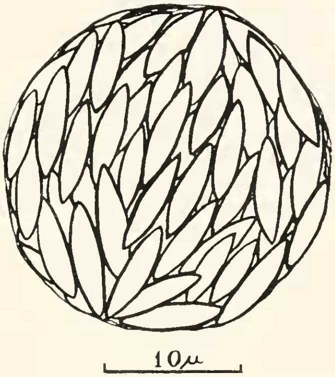

FIGURE 1. Aggregation of rodlets from glandular portion of nephridium of female.

Rodlets mounted in hanging drops of 100%, 80% and 60% S.W. were mostly intact after 5 hours but burst in that time in 40% S.W. In 20% S.W. all burst within 13 minutes and in distilled water within 3 minutes. In S.W. made alkaline to pH 11 with NaOH, in N H_2SO_4 and in S.W./N H_2SO_4 : 1/1, all burst quickly but in S.W. N H_2SO_4 : 4/1 most remained intact. In Bouin's fluid all the rodlets burst quickly but in 5% formalin in S.W. only some burst. Subsequent irrigation with distilled water caused them all to burst. Surface-active agents in sea water, both ionized (0.1% Teepol) and un-ionized (0.1% Triton × 100), caused the rodlets to burst. Teased male *Scoloplos* in S.W. had no visible effect. The conversion of the rodlets into jelly can thus be accelerated by various treatments but none of the tests throws much light on the natural process, which is rapid and once started goes to completion. Even in the tests with dilute S.W. there was never any suggestion of the rodlets taking up an intermediate size in equilibrium with a medium of osmotic pressure different from that of their normal environment.

Microscopical appearance

The cocoon jelly can be seen under the microscope (especially with phase-contrast) not to be perfectly homogeneous but somewhat fibrous in appearance. This also shows up in the fixed material stained with Heidenhain's Azan stain (blue), Mallory's stain (blue), the P.A.S. stain (red), muci-carmine (red) and

Alcian blue. Metachromasy is shown with toluidine blue. The fibrous appearance is in no way due to the presence of clearly defined fibers but rather to what are best described as local condensations of the jelly.

Chemical properties

The general chemical nature of the jelly is indicated by its staining properties. All these point to its being (or at any rate containing) a mucoprotein. Further tests were made in an attempt to find out details of its chemical composition and, if possible, to relate this to its physical properties and biological role.

Some 2000 cocoons were collected, teased up by hand, using needles, centrifuged at 5° C. for 20 minutes at 20,000 *g* and the jelly retained, the eggs and debris in the lower fraction being discarded. The process was repeated after further teasing of the jelly and a clean, egg-free jelly was obtained. Examined under the microscope it was seen to contain only scattered specks of unidentifiable material probably derived from the substratum. Any jelly that was not used fresh was stored in 50% isopropanol in a refrigerator until required.

Rough determinations of the organic content of the centrifuged native jelly were made by weighing a sample, washing it at least 8 times in distilled water until demonstrably chloride-free when tested with silver nitrate, and then drying to constant weight in air on a hot-plate. Values of the salt-free dry weight were 1.5% and 0.5% of the wet weight, the difference probably being due to the different amounts of the watery fluid which had been squeezed out by centrifugation before weighing, as well as to possible inequalities in drying. The jelly does not dissolve in sea water, distilled water or dilute acids. It dissolves in 0.5 N NaOH with gentle warming but it seems likely that this process brings about considerable degradation.

Nitrogen content

The nitrogen content of the jelly was estimated as follows. A sample was taken, washed in repeated changes of distilled water until the washings gave no trace of chloride when tested with silver nitrate solution. This was evaporated to dryness, washed again with distilled water and again evaporated to dryness. It was heated at 105° C. in an oven until of constant weight and two samples were used for total nitrogen estimation by the Kjeldahl method of Yuen and Pollard (1953). The average of two estimations gave a figure of 12.4% N (12.3% and 12.5%). This figure is below that normally accepted for protein (16%) and somewhat above that for an amino sugar such as glucosamine (7.8%). It looks, therefore, as if the material is a combination of protein and carbohydrate moieties.

Amino-acid composition

A sample of the dried material weighing 12.2 mg. was hydrolyzed by heating for 7 hours at 105° C. with 10 ml. of 6 N hydrochloric acid in a sealed tube. The solution was evaporated in a rotary film evaporator and was twice redissolved and evaporated almost to dryness. The residue was taken up in distilled water and left in an evacuated desiccator over calcium chloride and sodium hydroxide to remove the last traces of hydrochloric acid. Two-dimensional paper chromatography on Whatman No. 1, using butanol/acetic acid/water (12:3:5 by volume) and phenol/water (4:1 by weight) as solvents, showed that some 15 amino acids were present. They were mostly revealed by ninhydrin and identified by comparison with the positions taken up by known amino acids and also by the method of enhancement of each spot in turn by a known amino acid applied in addition to the hydrolysate. Runs were also made using as solvents ethanol/water/ammonia (12:1:1 by volume) and methanol/water/pyridine (20:5:1 by volume). The presence of aspartic acid, proline, hydroxyproline and alanine was confirmed by the use of isatin. The following amino acids were present: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, hydroxyproline, leucine (or isoleucine), lysine, methionine (or valine), ornithine, proline, serine, threonine, tyrosine. A feature of interest is the presence of hydroxyproline which is usually found only in collagenous tissue proteins. These frequently have mucopolysaccharides associated with them.

Carbohydrate components

Sugars were separated on Whatman No. 54 paper, in the first place by descending chromatography, using isopropanol/water (4:1 by volume). Runs were continued for 17 hours, which allowed the solvent to drip off the paper and increased the resolution of the spots with low R_f values. The sugars were revealed by silver nitrate in acetone, followed by sodium hydroxide in ethanol. The papers were bleached in ammonia and the spots blackened by hydrogen sulphide. The Elson-Morgan reagent for amino sugars was also used. These tests clearly showed the presence of glucosamine and/or galactosamine and traces of two other sugars. Additional runs in ethyl acetate/pyridine/water (12:5:4 by volume), followed by revelation with silver nitrate, showed one of the simple sugars to be glucose. The other corresponded in position to fucose (a hexose) and xylose (a pentose) which have similar R_f values in most solvents. Since the sugar was in low concentration in the hydrolysate the negative result of the phloroglucinol test for pentoses applied to the paper could not be relied upon to prove the absence of xylose. Accordingly the hydrolysate was tested for fucose, using the sulphuric acid/thioglycolic acid test for 6-deoxyhexoses (Dische, 1947), to which group of sugars fucose belongs. A positive result was obtained. Testing the hydrolysate for pentoses by Bials' orcinol reaction and the phloroglucinol test gave a negative result. From this it was concluded that the trace of sugar present in addition to glucose and revealed as having an R_f value indicating either fucose or xylose was, in fact, fucose.

The presence of both glucosamine and galactosamine was also confirmed by the ninhydrin degradation method of Stoffyn and Jeanloz (1954), followed by identification of the pentoses, arabinose and lyxose, which are produced by the reaction. This was done by paper chromatography, using ethyl acetate/pyridine/water as solvent, followed by revelation with silver nitrate and comparison with the position of known samples. The size and intensity of the spots obtained indicated that these two amino sugars were present in about equal amounts and in greater quantity than glucose and fucose.

Tests for uronic acids were made on the hydrolysate, using naphthoresorcinol. They were negative and confirmed the absence of any spot on the paper chromatogram with an R_f corresponding to that of uronic acids. The presence of sulphate groups was tested for by adding barium chloride solution to the hydrolysate. No precipitate was obtained and the sugars are therefore not sulphated.

Attempts were made to separate any possible constituents of the jelly by electrophoresis on paper and cellulose acetate membrane at pH 8.5 and pH 4.0 and 6 volts per cm., followed by detection with Schiff's reduced fuchsin sugar reagent and naphthalene black for proteins. All the tests failed to demonstrate any mobile component whether they were made on small blobs of dialyzed native jelly or on washings of the jelly concentrated by ultrafiltration through Visking tubing. All that can be concluded from this is that the molecular weight is very high or that the molecules have little net charge, but the results fit in with the other properties of the material, namely its insolubility and its extremely high viscosity at low concentration, which is suggestive of a cross-linked molecular structure.

An estimate by the anthrone method (Bangle and Alford, 1954) of the total quantity of simple sugars present in the organic component of the jelly was made, using a weighed, salt-free, dry sample. (This method gives no color with amino sugars in the concentrations present.) The intensity of color obtained was compared with that of standards from known concentrations of glucose in an EEL Spectra spectrophotometer at 625 mμ. The simple hexose component amounted to approximately 7% of the dry weight of the jelly.

From all these analytical procedures there is no doubt that the jelly can be called a mucoprotein having the carbohydrate moiety composed of galactosamine and glucosamine, glucose and fucose. One of the chief properties of such materials is their ability to bind large amounts of water to a very small amount of organic matter. The jelly of *Scoloplos* is no exception to this and was easily shown to be capable of imbibing water when dry and regaining its original volume or thereabouts. This process of drying and imbibition was repeated several times and was not affected by storage of the jelly in 50% isopropanol.

Physical properties

The bulk of the mucoprotein jelly does not dissolve in water but remains as a "blob" even after teasing and centrifugation. Moreover it cannot readily be dissolved by chemical methods which do not degrade it considerably. These properties are obviously of great importance in its role of egg container.

On the other hand its structure can be altered by certain purely physical means sufficiently to precipitate it or at least a component of it. When treated in a ground-glass testtube homogenizer, in which the jelly is subjected to considerable shearing forces, it yields a white precipitate which on drying becomes powdery and is unable to imbibe water and regain the original volume of the jelly. The liquid left after spinning off the precipitate was tested with the anthrone reagent and with the Elson-Morgan reagent for amino sugars. The anthrone test was positive but the Elson-Morgan gave only a very faint coloration. The anthrone method (Bangle and Alford, 1954) was then used to estimate the amount of hexose liberated from a sample of jelly by homogenization. By treatment in the homogenizer for various lengths of time up to 15 minutes, it was possible to split off about 5% of the dry weight of the jelly, corresponding to about 70% of the simple carbohydrate present.

From these tests it can be concluded that the gel-forming property of the material depends upon the presence of the carbohydrate component of the complex, and that this is only loosely attached and can be removed by physical means.

The tensile strength of the stalk is such that, when rapidly loaded in sea water at room temperature, it extends and breaks within seconds when a weight of about 8 g. is applied. When loaded with 1 g. or even 0.5 g. it stretches quickly at first and then more slowly but continues to extend slightly for 18 hours. Very slow extension was continuing when the experiment was stopped. When the tension was released there was an immediate shortening although not to the original length. These measurements were made using a smoked drum and light aluminum lever, and while it is clear that the jelly can be described in general terms as a viscous-elastic system, recording methods with less backlash and of greater sensitivity are needed to say how its properties may best be represented in a conventional way. In nature the cocoons may be expected to be under a slight stress during most of their life because, although the direction of the tide changes at ebb and flow, the cocoon stalk is flexible and so always takes up the same position with respect to the current. This stress is presumably less than the 0.5 g. which was shown to cause extension, but the sudden stresses to which the cocoons may be subjected by wave action would be expected to be much greater. For overcoming the effects of these the elastic element in the behavior would be effective since the forces act for only a short time.

A sample of the jelly was prepared for x-ray analysis by drying a film of the material on a glass plate. This film was peeled off and cut into rectangles 2 × 5 mm. which were stuck together by slight moistening to form a block 2 × 2 × 5 mm. The x-ray pattern perpendicular to the plane of the dried films of jelly showed disorientated protein but the x-ray pattern parallel to the dried films showed an indication of structure at the molecular level, composed of sheets separated by about 10 Å. It is tempting to suppose that these correspond to proteins which are cross-linked by the carbohydrates but further experiments are required before this can be established.

The Function of the Cocoons

The cocoon-forming habit occurs sporadically in the order Polychaeta and has thus presumably evolved many times. It is clearly based on the secretion of some part of the glandular epidermis which most annelids possess. Few authors have recorded the exact source of the cocoon jelly but the stage of hatching of the developing eggs is generally noted in the literature. It varies from the free-living trochophore, as in *Phyllodoce maculata*, or the metatrochophore, as in *Marphysa* sp., to chaetigerous larvae in *Aricia* spp. and *Scoloplos* spp.

There is no positive evidence that the laying of eggs in cocoons confers any nutritive, thermal or osmotic advantage, although the opinion has been expressed that because algae and Infusoria are frequently seen to flourish in the cocoon they provide a food supply for the larvae. Bookhout and Horn (1949) stated that the embryos of *Axiothella mucosa* fed on the diatom *Nitzschia* which multiplied in the cocoons, and Pillai (1958) is of the opinion that the embryos of *Marphysa borradailei* are nourished either by the jelly substance or the protozoans that flourish in it. It is possible that the larvae gain some nutriment from the jelly but it should be remembered that the eggs are generally packed with reserve food materials while the jelly contains only about 1% of organic material, and in *Scoloplos* and *Phyllodoce,* at any rate, the cocoons seem to be little altered when the larvae have left. Also, no evidence has been put forward to prove that the concentration of food organisms is greater in the jelly than in the substratum. In the locality in which *Scoloplos* and *Phyllodoce* occur at Southend-on-Sea, the surface of the sand is frequently much richer in diatoms than the interior of the cocoon jelly. The chief function of the cocoon appears to be to keep the young in their proper station but even this advantage is partly lost if trochophores are liberated and especially if they are positively phototactic like those of *Phyllodoce.* Further ecological studies are required to determine more precisely the role of the cocoons in the biology of their producers and what advantages, if any, the cocoon-laying habit confers.

I am grateful to Professor G. E. Newell, with whom many of the observations at Whitstable were made, to Dr. J. E. Forrest for information on the structure of the nephridia, and to Mr. A. G. Taylor who did much of the chemical analysis.

SUMMARY

1. The egg cocoons of *Scoloplos armiger* are laid at Whitstable during the few days of high spring tides in February or March.

2. The cocoon jelly is derived from secretion of cells forming the end portions of the nephridia of the female and contains only about 1% of organic matter which is mucoprotein. Fifteen amino acids, including hydroxyproline, were identified in the protein moiety and glucosamine, galactosamine, glucose and fucose in the carbohydrate portion.

3. The jelly can be dried but will swell again in water. This property can be destroyed by homogenization when 70% of the hexoses are split off.

4. It is unlikely that the cocoons play any significant part in the nutrition of the larvae, their chief function probably being to ensure that the larvae remain near a suitable substratum.

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